

Gene amplification and overexpression of *HER2* in renal cell carcinoma

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Objective To determine the frequency of *HER2* genetic abnormalities in renal cell carcinoma (RCC) and hence assess the potential suitability of Herceptin™ immunotherapy.

Patients and methods Tumours from 27 patients with RCC were assessed; all patients had undergone nephrectomy. Benign renal tissue from the nephrectomy specimens was studied in seven patients. Gene amplification was assessed using fluorescent *in-situ* hybridization, and protein over-expression using immunohistochemistry.

Results Twenty-four patients had clear cell renal carcinoma, two had papillary renal carcinoma and one a sarcomatoid variant carcinoma. There was no

HER2 amplification in the tumours or the benign renal tissue. Polysomy 17 was detected in 11 of 27 tumours (41%) and increased *HER2* copy number in seven (26%). Both polysomy 17 and increased *HER2* copy number were absent in the benign renal tissue. Three tumours (11%) and six of the seven benign renal tissue samples over-expressed the *HER2* protein.

Conclusions *HER2* amplification is absent and protein over-expression uncommon in RCC. This casts doubt on the suitability of Herceptin™ in the treatment of RCC.

Keywords renal carcinoma, *HER2*, Herceptin™, FISH, immunohistochemistry

Introduction

Patients with locally advanced or metastatic RCC have a poor prognosis; the 5-year survival for patients with stage IV disease is 10% [1], reflecting that RCC is largely resistant to conventional therapeutic regimens [2]. Immunotherapy using interleukin-2 and interferon- α combined has shown response rates of up to 18.6% in patients with metastatic disease, but is associated with significant side-effects [3]. Negrier *et al.* [3] reported grade 3 and 4 adverse events like hypotension (resistant to vasopressor agents) and fever in 45% and 35% of patients, respectively. A better understanding of the molecular genetics of RCC may help in the development of new therapeutic agents. Aneuploidy of chromosome 17 has been reported in papillary RCC [4,5]. The *HER2/c-erbB2* gene is located on chromosome 17 and encodes a transmembrane type I tyrosine kinase growth factor receptor with 50% homology with the EGFR [6–8]. Over-expression of *HER2* has been reported in breast [9–11], ovarian [9], lung [12] and salivary gland tumours [13], and is associated with a poorer prognosis. In a recent review examining the results of 47 studies involving >22 600 patients with breast cancer, *HER2*-positive status was an independent predictor of poor prognosis in multivariate analysis [14]. Herceptin™

(*rhu-mAb-HER2*, Trastuzumab, Genentech Inc., San Francisco, CA) is the humanized equivalent of the murine 4D5 mAb and is targeted against the *HER2* cell-surface receptor. Phase II and phase III clinical trials in patients with breast cancer over-expressing the *HER2* oncogene showed response rates of 11–15% [15,16]. A recent study showed that 40% of RCCs over-express the *HER2* protein and 17% show gene amplification [17,18]. However, other studies have not confirmed these findings, suggesting that the *HER2* expression and amplification rates are lower [19–21]. These differences may be partly explained by the different laboratory techniques used in the various studies. Determination of protein over-expression by immunohistochemistry (IHC) is highly dependent on the antibody selected and the methods used. Fluorescent *in-situ* hybridization (FISH) is the approved diagnostic method for determining gene amplification, allowing *in-situ* analysis of gene copy number. The Glasgow Royal Infirmary is one of three UK centres evaluating *HER2* status in breast cancer diagnosis [22]. In the present study we evaluated the *HER2* status of 27 RCCs and seven benign renal tissues using FISH and IHC.

Patients and methods

Tumours from patients with a pathological diagnosis of RCC and attending the Gartnavel General Hospital

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between 1997 and 1999 were included in the study. All patients underwent nephrectomy and tumours were assigned TNM [23] and Robson stages [24] and grades [25]. Benign (normal) renal tissue was identified from the RCC nephrectomy specimens. IHC was performed using the CB11 mouse mAb and the Nexus II automated staining system (both from Ventana, UK). Formalin-fixed paraffin-embedded 5 µm sections and positive/negative controls were used in each run. Antigens were retrieved in a pressure cooker for 12 min (1 L distilled water and 1.8 mmol of EDTA at pH 8 in an 850-W microwave oven), followed by a 20-min cooling period. Endogenous biotin was blocked by applying avidin for 15 min and then biotin for 15 min (SP-2001, Vector Laboratories, Burlingame, USA). The slides were transferred to the Nexus II and the basic diaminobenzidine detection kit (Ventana) applied; 100 µL of CB11 was added manually to each slide as appropriate and incubated in the Nexus II for 32 min. The result was scored by two independent observers. Routine sections stained with haematoxylin and eosin were examined to identify areas of tumour or benign renal tissue, and corresponding areas marked on the IHC sections. IHC was scored according to both the intensity and number of cells stained. No staining or membrane staining in <10% of cells was scored as 0; weak staining of the membrane in >10% of cells was scored as 1+ moderate membrane staining in >10% of cells was scored as 2+, and strong membrane staining in >10% of cells as 3+. Cytoplasmic staining was excluded. A score of 2+ or 3+ was regarded as 'positive' and a score of 0 or 1+ as 'negative' [26].

FISH was performed as previously described [27,28]; briefly, slides were pretreated with 0.2 mol/L HCl, 8% sodium thiosulphate at 80°C and 0.5% pepsin at 37°C. Sections were post-fixed in 10% neutral buffered formalin. The extent of digestion was estimated by applying 0.5 µg/mL 4,6-diamidino-2 phenylindole-2 hydrochloride in Vectashield (Vectorlabs, Peterborough, UK) antifade mounting medium. Sections were viewed at ×400 using a microscope. The target DNA was denatured with 100 µL of 70% formamide in 2× saline/citrate buffer, 10 µL of the probe applied and the slides incubated overnight in an Omnislide at 37°C (Hybaid, UK). Chromosome 17 and HER-2 copy number was evaluated using a direct dual-labelled technique, with the Pathvysion™ HER-2 DNA probe kit (Vysis, UK). This incorporated a Spectrum Green-labelled α-satellite probe for chromosome 17 and a Spectrum Orange-labelled HER2 probe. Again, serial sections stained with haematoxylin and eosin were first examined microscopically to locate tumour areas and benign renal tissue. FISH-stained sections were then scanned at ×400 to locate these regions. Three areas were identified and 20 nuclei assessed per area by two independent observers

with ×40 or ×100 objectives. Signals per nucleus for chromosome 17 (green) and HER2 (orange) were counted and the results recorded manually; in all, 60 nuclei were assessed. Gene amplification was defined as a HER2/chromosome 17 ratio of >2. Based on values from controls tissues in our laboratory, the normal values for chromosome 17 were 1.35–1.85 (1.61 ± 3 sd) and for HER2 were 1.51–1.99 (1.75 ± 3 sd). Values of >3 sd over the mean were defined as polysomic (for chromosome 17) or showing increased HER2 copy number.

Results

Twenty-seven tumours were assessed from 19 men and eight women; 24 tumours were of the clear cell type, two were papillary and one was of a spindle-cell variant. The clinical details of the 27 patients are summarized in Table 1, based on the TNM classification and more generally by the Robson classification (Table 2).

Table 1 Summary of the clinical details, FISH and IHC results of the 27 patients with RCC

Case/Sex	Grade	TNM	Copy number		IHC
			Chrom 17	HER2	
1/M	2	T3N0M0	1.51	1.68	0
2/F	2	T2N0M0	1.78	1.71	0
3/M*	4	T3N2M0	1.59	1.55	0
4/M	2	T1N0M0	1.85	1.69	0
5/M*	3	T3N0M0	2.66	2.88	0
6/F	3	T1N0M0	1.73	1.69	0
7/F	3	T2N0M0	2.5	2.88	0
8/F	4	T1N0M0	1.2	1.21	0
9/F	3	T3N0M0	1.73	1.74	2
10/M	2	T3N0M0	1.87	1.79	0
11/M	2	T2N0M0	1.19	1.18	0
12/M	3	T1N0M0	2.8	3.02	0
13/F	1	T3N0M0	1.71	1.63	0
14/M	3	T3NXM0	2.41	2.48	0
15/M	3	T3N1M0	1.9	1.78	0
16/M	3	T1N0M0	1.75	1.78	0
17/M	3	T2NXM0	1.58	1.69	0
18/M†	4	T4N0M0	2.03	1.65	0
19/M	3	T2N0M0	1.7	1.65	0
20/M	4	T3N0M0	2.16	2.2	0
21/M	2	T2N0M0	2.53	2.92	2
22/M	3	T1N0M0	1.74	1.87	2
23/M	2	T1NXM0	1.73	1.84	0
24/M	2	T3N0M0	2.32	2.37	0
25/M	3	T3N0M0	1.88	1.85	0
26/F	3	T2NXM0	1.83	1.71	1
27/F	3	T2NXM0	1.75	1.78	0

*Represents the two papillary tumours and †the spindle cell carcinoma.

Table 2 The distribution of stage and tumour type in the tumours in the study, and the distribution of the RCCs with polysomy 17 and increased HER-2 copy number; both these genetic events were more frequent in more advanced stages

Tumour type or result	Stage				N
	I	II	III	IV	
Clear cell	6	6	7	5	24
Papillary	0	0	1	1	2
Spindle cell variant	0	0	0	1	1
Total	6	6	8	7	27
Result:					
Polysomy 17	0	3	4	4	
Increased HER2 copy number	0	2	2	3	
IHC (2+ or 3+)	2	1	0	0	

All FISH hybridizations were successful; there was no gene amplification in any of the tumours or the benign renal tissue. The median (range) HER2/chromosome 17 number was 0.99 (0.8–1.17) for the tumours and 0.99 (0.93–1.15) for benign tissue. The median chromosome 17 copy number for the tumours was 1.78 (1.19–2.8) and 11 (41%) were polysomic for chromosome 17. The median chromosome 17 copy number for benign renal tissue was 1.59 (1.5–1.79) and hence none was polysomic for chromosome 17. The median HER2 copy number for the tumours was 1.76 (1.18–3.02) and hence seven (26%) had increased HER2 copy number (all seven were also polysomic for chromosome 17). The median HER2 copy number for benign renal tissue was 1.68 (1.4–1.8) and hence none had increased HER2 copy number.

All the IHC was completed successfully; three tumours (11%) over-expressed the HER2 protein (2+); all other tumours were negative. Six of the seven benign renal tissues over-expressed the HER2 protein (3+); all these positive samples had a particular distribution of positively staining cells, confined to the distal nephron (Fig. 1a,b).

Discussion

Patients with metastatic RCC have a 5-year survival of only 10% and RCC is resistant to conventional chemotherapeutic agents [1,2]. The study of the molecular genetics of RCC may help to develop new therapeutic agents. Alterations of genes that function as regulators of cell growth and differentiation are considered to be crucial in the progression of human cancers [29]. Numerous genetic aberrations have been documented in RCC and many are associated with a poor clinical outcome [30]. For example, the EGFR (on chromosome 7) is over-expressed in 80% and there is



Fig. 1. Positively staining: a, RCC tissue, scored as a '2+' and b, benign renal tissue; note the particular distribution of positively staining cells confined to the distal nephron in the latter. Both $\times 160$.

polysomy of chromosome 7 in 75% of RCCs, predicting a poor prognosis [31,32]. Polysomy 7 is linked to polysomy 17 in RCC [4,5] and the HER2 oncogene is on chromosome 17. Over-expression/gene amplification of HER2 has been reported for many human tumours and represents a poor prognostic factor in several [9,11–13]. HER2 gene amplification and protein over-expression are thought to be important in oncogenic transformation, tumorigenesis and metastasis [33]. For example, in breast cancer, patients whose tumours have gene amplification/protein over-expression (15–30% of all patients) have a poorer prognosis. These patients respond to clinical treatment with the mAb Herceptin, which is targeted against the HER2 cell surface receptor [15,16]. Herceptin has cytostatic growth inhibitory effects against breast cancer cells, and induces antibody-dependent cellular cytotoxicity against human tumour cell lines, contributing to its antitumour activity [33].

There is a wide variation in the reported results for HER2 assessment in RCC, partly because of the differing laboratory techniques applied in the various studies. Rotter *et al.* [18] used slot-blot analysis and found no gene amplification in any of 24 tumours. Similarly Weidner *et al.* [19] (six tumours), Freeman *et al.* [20] (13 tumours) and Stuum *et al.* [21] (34 tumours) showed no gene amplification by Southern blot analysis in RCCs. Classical techniques for detecting gene amplification, like Southern blotting, need a large quantity of DNA and all have the problem of varying tumour content within a given specimen, which renders the results unreliable if the percentage of tumours cells is low in a sample [10]. More recently Zhang *et al.* [17] (70 tumours) used differential PCR and reported that 17% of tumours showed gene amplification for HER2 in addition

to a significant association with tumour stage. The conclusion was that HER2 abnormalities may be involved in the pathogenesis of RCC. Differential PCR must be rigorously controlled if it is to be used successfully to quantify mRNA or gene copy number [34]. Furthermore, the use of tissue homogenates, as used for southern analyses (see above), may further compromise the sensitivity of this technique. FISH is the preferred technique for visualizing HER2 in breast cancer diagnostics, because it assesses DNA in individual cells using a specific fluorescently labelled probe. Hence there is no need to microdissect tumour cells or to isolate nucleic acids before labelling, as with Southern blotting, and indeed FISH has been shown to be superior to other techniques in detecting HER2 amplification on formalin-fixed, paraffin-embedded material [10]. Comparing the results of studies estimating protein over-expression of HER2 is difficult because all have used different antibodies and scoring criteria. However, Stuum *et al.* [21] and Zhang *et al.* [17] reported that 64% and 40% of RCCs over-expressed the HER2 protein using IHC, and that HER2 was important in the progression of RCC. In contrast, Rotter *et al.* [18] reported that HER2 protein over-expression was uncommon in RCC and that HER2 was not important in the progression of RCC. Consistent with this conclusion, others [19,20] reported that mRNA levels were lower in RCCs than in normal tissue. Hence the exact role of HER2 in the pathogenesis of RCC remains unclear.

In the present study of 27 RCCs and seven benign renal tissues examined for HER2 abnormalities using FISH and IHC, there was no gene amplification in any of the tissues. However, polysomy 17 occurred in 41% and increased HER2 copy number in 26% of the tumours. Furthermore, polysomy 17 and increased HER2 copy number were more frequent in stage III and stage IV cancers (Table 2). Both polysomy 17 and increased HER2 copy number were absent in the benign renal tissue.

Protein was over-expressed in 11% of the tumours at 2+ and in six of seven of the benign renal tissues at 3+. Hence protein was strongly over-expressed (3+) only in the benign renal tissue, confined to the distal parts of the nephron. There was no staining in the proximal tubules, which are the cells thought to give rise to RCC. This is consistent with results reported previously [19,20,35], suggesting that under-expression of HER2 mRNA is characteristic of RCCs. Consistent with this, Rotter *et al.* [18] suggested that reduced HER2 protein and gene expression were inversely related to tumour differentiation, with less well differentiated tumour cells having lower HER2 expression. Current evidence suggests that in breast cancer only patients with tumours that are 3+ on IHC or 2+ with gene

amplification, as shown by FISH, respond to Herceptin immunotherapy [36], implying that the advanced RCCs in the current study may be unresponsive to Herceptin.

The present results suggest that gene amplification and strong protein over-expression of HER2 is absent in RCC. This casts doubt on the suitability of Herceptin immunotherapy in the management of RCC.

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References

- 1 Selli C, Hinshaw WM, Woodard BH, Paulson DF. Stratification of risk factors in renal cell carcinoma. *Cancer* 1983; 52: 899-903
- 2 Yagoda A, Abi-Rached B, Petrylak D. Chemotherapy for advanced renal-cell carcinoma: 1983-93. *Semin Oncol* 1995; 22: 42-60
- 3 Negrier S, Escudier B, Lasset C *et al.* Recombinant human interleukin-2, recombinant human interferon alfa-2a, or both in metastatic renal cell carcinoma. *New Engl J Med* 1998; 338: 1272-8
- 4 Corless CL, Aburatani H, Fletcher JA, Houseman DE, Ami MB, Weinberg DS. Papillary renal cell carcinoma. Quantitation of chromosomes 7 and 17 by FISH, analysis of chromosome 3p for LOH, and DNA ploidy. *Diag Mol Pathol* 1996; 5: 53-64
- 5 Brown JA, Borell TJ, Qian J, Bostwick DG, Jenkins RB. Simultaneous chromosome 7 and 17 gain and sex chromosome loss provide evidence that metanephric adenoma is related to papillary renal cell carcinoma. *J Urol* 1997; 158: 370-4
- 6 Schechter AL, Hung MC, Vaidyanathan L *et al.* The neu gene. An erbB-homologous gene distinct from and unlinked to the gene encoding the EGF receptor. *Science* 1985; 229: 976-8
- 7 Bargmann CI, Hung MC, Weinberg RA. The neu oncogene encodes An epidermal growth factor receptor-related protein. *Nature* 1986; 319: 226-30
- 8 Akiyama T, Sudo C, Ogawara H, Toyoshima K, Yamamoto T. The product of the human c-erbB-2 gene: a 185-kilodalton glycoprotein with tyrosine kinase activity. *Science* 1986; 232: 1644-6
- 9 Slamon DJ, Godolphin W, Jones LA *et al.* Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* 1989; 244: 707-12
- 10 Pauletti G, Godolphin W, Press MF, Slamon DJ. Detection and quantitation of HER-2/neu gene amplification in human breast cancer archival material using fluorescence *in situ* hybridization. *Oncogene* 1996; 13: 63-72

- 11 Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer. Correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* 1987; 235: 177-82
- 12 Schneider PM, Hung MC, Chiocca SM *et al.* Differential expression of the c-erbB-2 gene in human small cell and non-small cell lung cancer. *Cancer Res* 1989; 49: 4968-71
- 13 Press MF, Pike MC, Hung G *et al.* Amplification and overexpression of HER-2/neu in carcinomas of the salivary gland: correlation with poor prognosis. *Cancer Res* 1994; 54: 5675-82
- 14 Revillion F, Bonnetterre J, Peyrat JP. ERBB2 oncogene in human breast cancer and its clinical significance. *Eur J Can* 1998; 34: 791-808
- 15 Cobleigh MA, Vogel CL, Tripathy D *et al.* Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. *J Clin Oncol* 1999; 17: 2639-48
- 16 Baselga J, Tripathy D, Mendelsohn J *et al.* Phase II study of weekly intravenous recombinant humanized anti-p185HER2 monoclonal antibody in patients with HER2/neu-overexpressing metastatic breast cancer. *J Clin Oncol* 1996; 14: 737-44
- 17 Zhang XH, Takenaka I, Sato C, Sakamoto H. p53 and HER-2 alterations in renal cell carcinoma. *Urology* 1997; 50: 636-42
- 18 Rotter M, Block T, Busch R, Thanner S, Hoffer H. Expression of HER-2/neu in renal-cell carcinoma. Correlation with histologic subtypes and differentiation. *Int J Cancer* 1992; 52: 213-7
- 19 Weidner U, Peter S, Strohmeier T, Hussnatter R, Ackermann R, Sies H. Inverse relationship of epidermal growth factor receptor and HER2/neu gene expression in human renal cell carcinoma. *Cancer Res* 1990; 50: 4504-9
- 20 Freeman MR, Washecka R, Chung LW. Aberrant expression of epidermal growth factor receptor and HER-2 (erbB-2) messenger RNAs in human renal cancers. *Cancer Res* 1989; 49: 6221-5
- 21 Stumm G, Eberwein S, Rostock-Wolf S *et al.* Concomitant overexpression of the EGFR and erbB-2 genes in renal cell carcinoma (RCC) is correlated with dedifferentiation and metastasis. *Int J Cancer* 1996; 69: 17-22
- 22 Ellis IO, Dowsett M, Bartlett J *et al.* Recommendations for HER-2 testing in the UK. *J Clin Pathol* 2000; 53: 890-2
- 23 Hermanek P, Hutter RVP, Sobin LH, Wagner G, Wittekind Ch. *TNM Atlas*. 4th edn. International Union against Cancer, 1997, 296-302
- 24 Robson CJ, Churchill BM, Anderson W. The results of radical nephrectomy for renal cell carcinoma. *J Urol* 1969; 101: 297-301
- 25 Fuhrman SA, Lasky LC, Limas C. Prognostic significance of morphologic parameters in renal cell carcinoma. *Am J Surg Pathol* 1982; 6: 655-63
- 26 Van de Vijver MJ. Assessment of the need and appropriate method for testing for the human epidermal growth factor receptor-2 (HER2). *Eur J Can* 2001; 37: S11-7
- 27 Bartlett JMS, Watters AD, Ballantyne SA, Going JJ, Grigor KM, Cooke TG. Is chromosome 9 loss a marker of disease recurrence in transitional cell carcinoma of the urinary bladder? *Br J Cancer* 1998; 77: 2193-8
- 28 Watters AD, Ballantyne SA, Going JJ, Grigor KM, Bartlett JMS. Aneusomy of chromosomes 7 and 17 predicts the recurrence of transitional cell carcinoma of the urinary bladder. *BJU Int* 2000; 85: 42-7
- 29 Strohmeier GT, Slamon DJ. Proto-oncogenes and tumour suppressor genes in human urological malignancies. *J Urol* 1994; 151: 1479-97
- 30 Moch H, Presti JC, Sauter G *et al.* Genetic aberrations detected by comparative genomic hybridisation are associated with clinical outcome in renal cell carcinoma. *Cancer Res* 1996; 56: 27-30
- 31 Moch H, Sauter G, Bucholz N, Waldman FM, Mihatsch MJ. Epidermal growth factor receptor expression is associated with rapid tumour cell proliferation in renal cell carcinoma. *Human Pathol* 1997; 28: 1255-9
- 32 Moch H, Sauter G, Gasser TC *et al.* EGF-r gene copy number changes in renal cell carcinoma detected by FISH. *J Pathol* 1998; 184: 424-9
- 33 Baselga J. Clinical trials of Herceptin (trastuzumab). *Eur J Can* 2001; 37: S18-24
- 34 Bartlett JMS, Hulme MJ, Miller WR. Analysis of Camp RI alpha mRNA expression in breast cancer. Evaluation of quantitative PCR for routine use. *Br J Cancer* 1996; 73: 1538-44
- 35 Yao M, Shuin T, Misaki H, Kubota Y. Enhanced expression of c-myc and epidermal growth factor receptor genes in primary human renal cell cancer. *Cancer Res* 1988; 48: 6753-7
- 36 Piccart M. Closing remarks and treatment guidelines. *Eur J Can* 2001; 37: 30-3

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Abbreviations: FISH, fluorescent *in-situ* hybridization;
 IHC, immunohistochemistry.